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Appraisal of Interpretation Criteria for the Comparative Intradermal Tuberculin Test for Diagnosis of Tuberculosis in Cattle in Central Ethiopia▽

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Accurate detection and removal of infected cattle, using immunodiagnostic tests such as the comparative intradermal tuberculin (CIDT) test, are the basis of control strategies for bovine tuberculosis (TB). According to the Office des Internationale Epizooties recommendation, the cutoff point for positivity of the CIDT test, calculated as the difference between skin thicknesses after bovine tuberculin (*B*) and avian tuberculin (*A*) injections (*B* − *A*), is >4 mm. This cutoff point is used worldwide, although it is likely that local conditions influence test performance. Thus, this study was formulated to determine CIDT test cutoff points applicable to cattle in central Ethiopia. Receiver operating characteristic analysis was performed for the CIDT test, using data from 186 *Bos indicus* (zebu) and *Bos taurus* (Holstein) cattle. Detailed postmortem examination for the presence of TB lesions was used to define disease status. At a cutoff of >2 mm, CIDT test sensitivity was 69% (95% confidence interval [95% CI], 58.5 to 79%), while it was 59% (95% CI, 49 to 69%) at a cutoff of >4 mm. In contrast, specificities of the CIDT test at these two cutoff values were identical, at 97% (95% CI, 89 to 100%). Thus, the maximum sensitivity of the CIDT test can be realized using a >2-mm cutoff without affecting specificity. The apparent prevalence was significantly ($\chi^2 = 13.56$; $P < 0.001$) higher at a cutoff of >2 mm (16.0%; $n = 5{,}424$) than at a >4-mm cutoff (13.5%; $n = 5{,}424$). Nonetheless, no significant difference ($\chi^2 = 2.15$; $P = 0.14$) in true prevalence was observed at a cutoff of >2 mm (19.6%) and at a cutoff of >4 mm (18.5%). Thus, our study demonstrates the importance of defining local, relevant cutoff values to maximize test sensitivity, and we suggest the application of the >2-mm cutoff for testing of cattle in central Ethiopia.

The tuberculin skin test is the primary diagnostic test for tuberculosis (TB) in both humans and cattle. Tuberculins are crude antigen preparations derived from heat-killed cultures of mycobacteria and contain mixtures of proteins, polypeptides, nucleic acids, and substantial amounts of polysaccharides (4). The comparative intradermal tuberculin (CIDT) test involves the intradermal injection of tuberculins, purified protein derivatives (PPDs) from *Mycobacterium bovis* and *Mycobacterium avium*, and the subsequent detection of swelling and indurations at the injection site 72 h later. The relative change in skin thickness at the two sites is used to differentiate *M. bovis* infection from infection with nontuberculous mycobacteria. According to the Office des Internationale Epizooties (OIE) (27) recommendation, the difference between the increase in skin thickness following the intradermal administration of bovine PPD (*B*) and the increase in skin thickness following the intradermal administration of avian PPD (*A*), *B* − *A*, should be >4 mm for the animal to be considered positive for bovine TB (bTB). Thus, the OIE advocates the use of this cutoff point for the diagnosis of bTB in live animals. Nonetheless, different cutoff values are applied according to a particular country's disease status and the objective of its disease control program. Moreover, because the OIE cutoff value was established mainly in developed countries for *Bos taurus* cattle, it should be reevaluated in *Bos indicus* and in *Bos taurus* cattle under different environmental conditions. In particular, the tuberculin skin test performance could be affected by environmental factors, the prevalence of TB, host factors, (status of immunity, genetics, etc.), and the nature of the tuberculin used (5). Therefore, the cutoff point ideal for one group in a specific geographic area may be less ideal for another group in another environment (32).

In this study, the cutoff point of the CIDT test was assessed with the Ethiopian Arsi breed, which belongs to *Bos indicus* (zebu), and also with a mixed population of Holstein, *Bos taurus*, and Arsi breeds grazing together under identical husbandry conditions, using receiver operating characteristic (ROC) analysis. The standard used to define disease status was the result of postmortem examination, which involved detailed gross examination of the head, thoracic and abdominal lymph nodes, and lungs, using inspection, palpation, and incision of suspicious tissues into pieces for further visualization. In addition, the true prevalence of bTB in 5,424 cattle was established by using the cutoff values of the CIDT test defined in this study.

MATERIALS AND METHODS

Study cattle. For ROC analysis, 161 Arsi and 25 Holstein cattle were recruited from grazing herds that were kept under identical traditional cattle management

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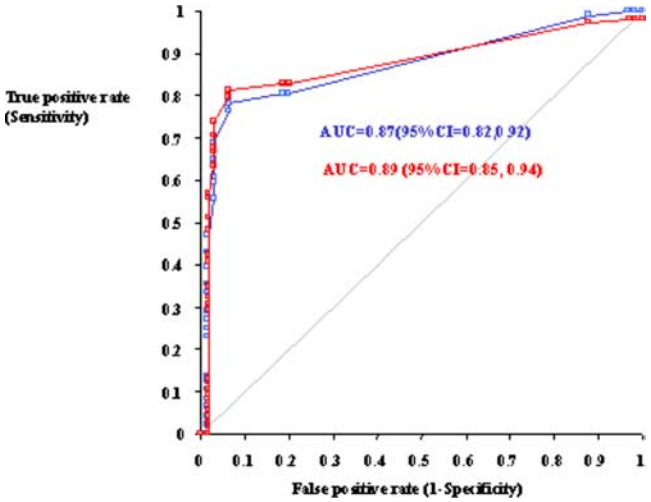

FIG. 1. ROC curves for the CIDT test in zebu cattle (blue line and symbols) and Arsi and Holstein cattle (red line and symbols). The ROC plots pass through the upper left corner, and the areas under the ROC curves are 0.87 and 0.89, for Arsi cattle and Arsi and Holstein cattle, respectively. Blue letters, Arsi cattle; red letters, Arsi and Holstein cattle.

in central Ethiopia. The prevalence of bTB was assessed by using different cutoff points for a mixed population of a total of 5,424 Arsi, Arsi × Holstein, and Holstein cattle that were kept together in central Ethiopia; females were used for milk production, while males were used for plowing.

CIDT test. Two sites on the right side of the mid-neck, 12 cm apart, were shaved, and the skin thicknesses were measured with calipers. One site was injected with an aliquot of 0.1 ml containing 2,500 IU/ml bovine PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom). Similarly, 0.1 ml of 2,500-IU/ml avian PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom) was injected into the second site. After 72 h, the skin thicknesses at the injection sites were measured. Cutoff points were assessed using ROC analysis.

Postmortem examination. The lungs and lymph nodes were removed for the investigation of gross pathological lesions. The seven lobes of the two lungs, namely, the (i) left apical, (ii) left cardiac, (iii) left diaphragmatic, (iv) right apical, (v) right cardiac, (vi) right diaphragmatic, and (vii) right accessory lobes, were inspected externally and palpated. Each lobe was then sectioned into about 2-cm-thick slices to facilitate the detection of lesions. Similarly, lymph nodes, namely, the (i) mandibular, (ii) medial retropharyngeal, (iii) cranial and caudal mediastinal, (iv) left and right bronchial, (v) hepatic, and (vi) mesenteric lymph nodes, were sliced into thin sections (circa 2 mm thick) and inspected for the presence of visible lesions. When gross lesions suggestive of bTB were found in any of the tissues examined, the animal was classified as lesioned. Animals in which lesions were not found were classified as nonlesioned.

Statistical analysis. ROC analysis was performed using Analyze-it software (Analyze-it Ltd., Leeds, United Kingdom) as an add-on to Microsoft Excel. Logistic regression analysis was used to assess the association between prevalence and animal risk factors, including age and breed, using STATA statistical software (Stata Corporation, College Station, TX). The difference in prevalence of bTB between breeds was analyzed using the EPI6 dose version and was compared using the Pearson chi-square (χ^2) test. Odds ratios were calculated to assess the strength of association of animal risk factor (age and breed) and the prevalence of bTB.

RESULTS

ROC curve of CIDT test in zebus. To reevaluate the CIDT test cutoff point in a local Ethiopian context, ROC analysis was performed using data from 161 Arsi cattle. These animals were subjected to skin testing and then slaughtered, and detailed postmortem analysis was performed to define their disease status. The ROC curve is depicted in Fig. 1 (blue line). The area under the ROC curve (Fig. 1) was 0.87 (95% confidence interval [95% CI] = 0.82 to 0.92). Sensitivity and specificity were also recorded in relation to different values of the tabulated $B - A$ differential (Table 1). For example, at a cutoff value of >2 mm, the sensitivity was 69% (95% CI = 58.5% to 79%), while the specificity was 97% (95% CI = 89% to 100%). On the other hand, applying the OIE recommended cutoff of >4 mm, the sensitivity was lower, at 59% (95% CI = 49% to 69%), without a gain in specificity compared to that with the >2-mm cutoff value. The difference in sensitivities of the CIDT test at a cutoff value of >2 mm and a cutoff of >4 mm was marginally significant ($\chi^2 = 3.45$; $P = 0.05$). Thus, a cutoff of >2 mm could be applied without a loss of specificity compared with that of the OIE-recommended >4-mm cutoff. Reducing the cutoff further resulted in a loss of specificity (Table 1).

It would have been of interest to perform a similar ROC analysis with Holstein cows held in central Ethiopia. However, this was not possible because Holstein cattle, due to their immense value, are rarely sold, and skin test-negative animals were therefore unavailable for postmortem analysis. Thus, a separate ROC analysis for Holstein cattle was not possible. Therefore, we combined the data from a small set of skin test-positive Holstein cows that we were able to examine postmortem with the results from the Arsi cattle in an additional ROC analysis to determine if this would change the result significantly, i.e., suggest that different cutoffs should be applied for Holsteins and zebus. The results in Fig. 1 (red line) suggested that this was not the case, as the curve did not change and there were also almost identical areas under the curves, i.e., 0.89 (95% CI = 0.85 to 0.94) for the combined Holstein and Arsi cattle and 0.87 (95% CI = 0.82 to 0.92) for Arsi cattle alone.

Apparent prevalence of bTB at cutoff points of >2 mm and >4 mm in zebu and Holstein cattle. In an earlier study, we assessed the skin test prevalence of bTB in 5,424 cattle consisting of zebus, Holsteins, and their crosses, using the OIE-recommended cutoff value of >4 mm (2). Based on the results described in the previous section, we reanalyzed these data using a >2-mm cutoff point. The apparent prevalence at the >2-mm cutoff point was 16.0% (868/5,424 cattle), compared to 13.5% (732/5,424 cattle) at the >4-mm cutoff point. Confirming and extending our earlier results (2) for both cutoff points, the apparent prevalence was significantly higher in Holsteins

TABLE 1. Sensitivities, specificities, and cutoff points of CIDT test for cattle in central Ethiopia[a]

Cutoff value (mm)	Sensitivity (95% CI)	Specificity (95% CI)
0.0	0.802 (0.708–0.876)	0.800 (0.682–0.889)
0.5	0.802 (0.708–0.876)	0.815 (0.700–0.901)
1.0	0.781 (0.685–0.859)	0.938 (0.850–0.983)
1.5	0.760 (0.663–0.842)	0.938 (0.850–0.983)
2.0	**0.688 (0.585–0.778)**	**0.969 (0.893–0.996)**
3.0	**0.646 (0.542–0.741)**	**0.969 (0.893–0.996)**
3.5	**0.604 (0.499–0.703)**	**0.969 (0.893–0.996)**
4.0	**0.594 (0.489–0.693)**	**0.969 (0.893–0.996)**
4.5	0.552 (0.447–0.654)	0.969 (0.893–0.996)
5.0	0.469 (0.366–0.573)	0.985 (0.917–1.000)

[a] Cutoff values ranging between >2 mm and 4 mm (shown in bold) gave optimal sensitivities and specificities.

TABLE 2. Association of host-related risk factors to skin test positivity at cutoff points of >4 mm and >2 mm for TB in central Ethiopia[a]

Factor	No. of cattle examined	No. (%) positive for TB	χ^2 value[b]	*P* value[b]
Cutoff of >4 mm[a]				
Breed			71.87	<0.001
Arsi (zebu)	2,578	298 (11.6)		
Cross	1,921	229 (11.9)		
Holstein	925	205 (22.2)		
Age (yr)			51.18	<0.001
<2	892	73 (8.2)		
2–5	1,868	237 (12.7)		
5–9	1,792	317 (17.7)		
>9	872	105 (12.0)		
Cutoff of >2 mm				
Breed			65.50	<0.001
Arsi (zebu)	2,578	358 (13.9)		
Cross	1,921	280 (14.6)		
Holstein	925	230 (24.9)		
Age (yr)			45.05	<0.001
<2	892	96 (10.8)		
2–5	1,868	289 (15.5)		
5–9	1,792	362 (20.2)		
>9	872	121 (13.9)		

[a] Another version of results applying to the >4-mm cutoff value was reported earlier (2), and in this case, the data were extracted from the paper for reasons of comparison.

[b] The chi-square (χ^2) test was used for the determination of statistical significance. *P* values of <0.05 were considered statistically significant.

than in zebus either at a cutoff of >4 mm (22.2% versus 11.6% [$\chi^2 = 61.8$; $P < 0.001$]) or at a cutoff of >2 mm (24.9% versus 13.9% [$\chi^2 = 65.5$; $P < 0.001$]) (Table 2). Moreover, at both cutoff points, significant differences ($P < 0.001$) in prevalence were observed among the different age groups (Table 2). The difference in apparent prevalence rates between the two cutoffs was statistically significant (16.0% versus 13.5% [$\chi^2 = 13.56$; $P < 0.001$]) (Table 3).

True prevalence of bTB at >2-mm and >4-mm cutoff points. Apparent prevalence may underestimate the true prevalence of a disease because the test used to determine apparent prevalence does not capture all infected animals (30). Hence, the true prevalence can be calculated using the following formula described by Rogan and Gladen (30): TP = (AP + SP − 1)/(SE + SP − 1), where TP is true prevalence, AP is apparent prevalence, SE is sensitivity, and SP is specificity.

Thus, the true prevalence was calculated (Table 3) using the sensitivities and specificities calculated by the ROC analysis. Accordingly, the true prevalence at a cutoff of >4 mm was 18.5%, while it was 19.6% at a cutoff of >2 mm. There was no significant ($\chi^2 = 2.15$; $P = 0.14$) (Table 3) difference between the true prevalence rates at the two cutoff points, and it can therefore be assumed that the true prevalence of bTB in the study population was around 19.0%.

DISCUSSION

In this study, postmortem examination was used to define disease status, as opposed to culture, which is a gold standard. A tentative diagnosis of bTB can be made by detection of macroscopic lesions at necropsy. The sensitivity of gross postmortem examination is affected by the method employed and the anatomical sites examined; careful examination of as few as six pairs of lymph nodes, the lungs, and the mesenteric lymph nodes can result in detection of 95% of cattle with macroscopic lesions (9). In the present study, detail postmortem examination, as described in Materials and Methods, i.e., examination of more than six pairs of lymph nodes and the lungs, was performed for each of the study animals. Thus, the method of inspection used in this study is superior to routine abattoir inspection procedures and thus can be used for the definition of disease status. Nevertheless, the use of postmortem examination as a gold standard for the determination of the optimal cutoff value of the CIDT test is acknowledged.

The cornerstone of TB control in cattle is the accurate detection and removal of infected cattle; however, because the infection is usually chronic and can remain subclinical for a long period, infected cattle can become infectious long before they exhibit any obvious clinical signs. As a result, effective antemortem surveillance must rely primarily on the detection of infected cattle at an early stage by the use of sensitive immunodiagnostic tests (1). Although imperfect, no better general approach for TB screening of cattle populations has been devised since Robert Koch discovered tuberculin (19). Programs based on the basic principles of systematic and regular skin testing of cattle herds, supplemented with compulsory removal of test reactors, movement restriction of known infected herds, and slaughterhouse surveillance of undetected infection, have eradicated bTB from many developed countries (6, 8, 10, 11).

The diagnostic accuracy of a test is defined primarily in terms of its sensitivity and specificity. There is an inverse relationship between test sensitivity and specificity (23), and these two parameters are frequently assumed to be constant across different populations as long as the test procedure and cutoff point for a positive test result remain the same from one group of animals to another (12). However, for TB diagnostics, this is not necessarily the case, as test results are influenced by the stage and severity of the disease (25, 31). This was the

TABLE 3. Comparison of apparent and true prevalence rates of bTB in central Ethiopia at cutoffs of >4 mm and >2 mm[a]

Cutoff point (mm)	% Sensitivity (95% CI)	% Specificity (95% CI)	No. of positive cattle/no. of cattle examined	Apparent prevalence (%)	Difference in apparent prevalence (χ^2 value, *P* value)	True prevalence (%)	Difference in true prevalence (χ^2 value, *P* value)
>2	68.8 (58.5, 77.8)	96.9 (89.3, 99.9)	732/5,424	16.0	13.56, 0.001	19.6	2.15, 0.14
>4	59.4 (48.9, 69.3)	96.9 (89.3, 99.9)	868/5,424	13.5		18.5	

[a] Sensitivities, specificities, and apparent prevalence rates were used to determine the true prevalence rates according to the formula described by Rogan and Gladen (30).

reason that we initiated a reevaluation of the OIE-recommended cutoff (>4 mm) in an Ethiopian context, using ROC analysis. Our data showed that sensitivities and specificities of the CIDT test were optimal at cutoff values ranging from >2 mm to >4 mm for zebu cattle in central Ethiopia. At a cutoff of >2 mm, the CIDT test's sensitivity was 69%, while it was 59% at a cutoff of >4 mm, with identical specificities, at 97%, for the two cutoff points.

ROC analysis is used to visualize, organize, and select classifiers based on their performance (14, 15, 34) and has been extended for use in visualizing and analyzing the behavior of diagnostic systems (33). Our results showed that the ROC plots for the CIDT test are high in the left corner, indicating good performance of the CIDT test for both the zebu breed and a mixed population of zebus and Holsteins. The area under the curve was 0.87 for zebus, while it was 0.89 for the mixed population of zebus and Holsteins, demonstrating moderate to high discriminatory power of the CIDT test.

The published sensitivity estimates for the tuberculin test were summarized by de la Rua-Domenech et al. (12), and the estimates ranged from 63.2% to 100%, with a median sensitivity of 83.9%, for the single intradermal tuberculin test and from 52.0% to 100%, with a median value of 80.0%, for the CIDT test. The sensitivity recorded by the present study is relatively lower than the median sensitivity (80.0%) of the CIDT test reported by other authors but is well within the reported range.

There are different reasons which could cause false-negative results, lowering the sensitivity of the CIDT test. For instance, newly infected animals may not react to the CIDT test, as reactions have been reported to develop between 3 and 6 weeks postinfection for most animals (16, 17, 21). A state of anergy can also develop in animals with advanced or generalized TB and in animals subjected to stress (22, 28), such as calving within the preceding 4 to 6 weeks (24). In addition, administration of glucocorticoids can also lead to lower indurations of tuberculin reactions in infected animals (13), while coinfection with viruses such as bovine viral diarrhea virus could transiently compromise the reaction to tuberculin (7). Furthermore, a reduced tuberculin reaction can occur if the infected animal is malnourished (24). The phenomenon of desensitization, which describes the depressed skin reactivity to the second tuberculin injection in naturally and experimentally infected cattle for some time after the first tuberculin injection, can also reduce the sensitivity of the tuberculin test (18, 20, 24, 29). Furthermore, prior tuberculin test exposure to mycobacteria of the *M. avium* complex and/or the *Mycobacterium intracellulare* complex may also lower sensitivity, as the reaction to avian tuberculin could be high and thus interfere with the interpretation of the result (17).

In our study, we found antibodies for paratuberculosis in 4% ($n = 263$) of animals (G. Ameni and M. Vordermeier, unpublished data), using the CSL Paracheck enzyme-linked immunosorbent assay with randomly selected sera obtained from the same population used as sources of our sampled cattle for the ROC analysis. In addition, we found an 80% ($n = 263$) (Ameni and Vordermeier, unpublished data) seroprevalence of *Fasciola hepatica* in the same group of animals by enzyme-linked immunosorbent assay at the Veterinary Laboratories Agency. Therefore, the relatively lower sensitivity recorded by our ROC analysis could also be attributed to coinfections with *Fasciola hepatica* and/or *M. avium* subsp. *paratuberculosis*, which could interfere with and compromise the response to tuberculin.

Using our data set, we calculated the apparent prevalence rates with the two cutoff values following ROC analysis to see the level of difference in apparent prevalence rates at the two cutoff points. Apparent prevalence is calculated as the number of test-positive animals divided by the total number of animals tested (23). We found a significant difference in apparent prevalence rates at the two cutoff points. Although it is useful as a consistent index and is used routinely to measure disease frequencies, apparent prevalence may underestimate the true prevalence of disease because the test used to determine apparent prevalence does not capture all infected animals (26, 30). The true prevalence of a disease in a population can be calculated using the apparent prevalence (as detected by some screening test) together with the sensitivity and specificity values of the test (30). Comparison of the true prevalence rates at the two cutoff points showed no significant difference. This is because, in addition to sensitivity and apparent prevalence, the calculation of true prevalence takes specificity into account. But in this case, although there were increases in sensitivity and apparent prevalence at a cutoff of >2 mm, the specificity was the same at both cutoff values, and the increase in true prevalence at a cutoff of >2 mm was not significantly different from the true prevalence at a cutoff of >4 mm.

Confirming our previous report (2) on both cutoff points, the apparent prevalence was significantly higher in Holsteins than in either crosses or zebus kept under identical husbandry conditions. This difference in breed susceptibility was also highlighted by the demonstration of increased disease severity in Holsteins (3).

In conclusion, our study emphasizes the need to define local cutoff values to ensure maximum test sensitivity to detect TB in cattle. In particular, we suggest that a >2-mm cutoff be applied to both breeds in central Ethiopia, rather than the OIE-recommended >4-mm cutoff, resulting in increased sensitivity without a loss of specificity.

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